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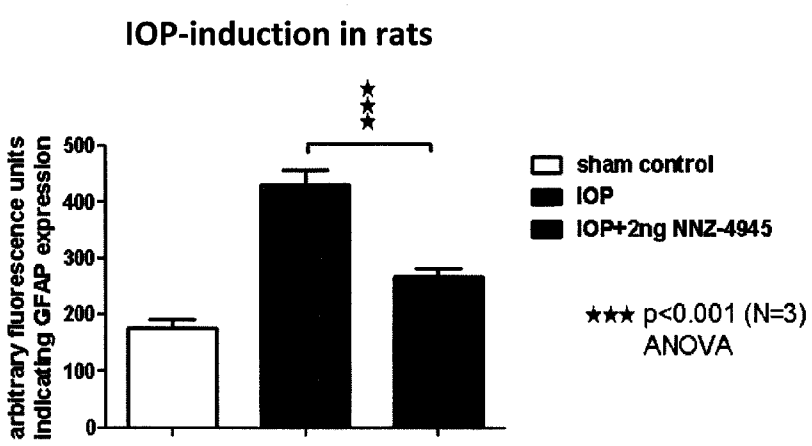
(54) Title: COMPOSITIONS FOR ADMINISTRATION TO THE EYE

FIGURE 13



(57) Abstract: The invention relates to methods and compositions for use in the prevention, treatment or reduction of retinal hypoxia and intraocular pressure associated retinopathy in subjects using an effective amount of one of the peptides: GlyArgArgAlaAlaProGlyArgAibGlyGly (SEQ ID NO:1)or GlyArgArgAlaAlaProGlyArgAibGlyGly-NH2 (SEQ ID N0:2).

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COMPOSITIONS FOR ADMINISTRATION TO THE EYE

FIELD OF INVENTION

The invention relates to the treatment of retinopathies. In particular, the invention relates to methods and compositions for use in the treatment of retinal hypoxia and intraocular pressure associated retinopathy.

BACKGROUND ART

Neuronal Regeneration Peptides (NRPs) are oligopeptides that exhibit neuroprotective, neurogenic, neuro-migratory and neuronal differentiating promoting-activity to neural progenitors, neuroblasts and differentiated neurons. The first published NRP sequence to be located in the human genome was part of the N-terminal length (NRP-derived position 38-50) of a protein, named CAPS-2 (calcium-dependent activator protein of secretion-2, which has been linked to the regulation of Brain Derived Neurotrophic Factor (BDNF) and Neurotrophin-3 (NT-3) (Sadakata *et al* (2004); Sieg and Antonic (2007)).

In vitro work has shown this family of peptides to be able to promote migration, neurite outgrowth, synaptogenesis and cell survival in a population of cultured primary cerebellar cells (Sieg and Antonic (2007)).

STATEMENT OF INVENTION

In a first aspect the invention provides a method of preventing, treating or reducing atrophy of a retina in a subject, comprising the step of administration to an eye of the subject an effective amount of at least one of the peptides:

GlyArgArgAlaAlaProGlyArgAibGlyGly (SEQ ID NO:1)or

GlyArgArgAlaAlaProGlyArgAibGlyGly-NH_2(SEQ ID NO:2).

In one embodiment the atrophy is caused by a hypoxic or ischemic insult.

In a second aspect the invention provides a method of preventing, reducing or treating intraocular pressure associated atrophy of a retina in a subject comprising the step of administration to an eye of the subject an effective amount of at least one of the peptides:

GlyArgArgAlaAlaProGlyArgAibGlyGly (SEQ ID NO:1)or

GlyArgArgAlaAlaProGlyArgAibGlyGly-NH_2 (SEQ ID NO:2).

In a third aspect the invention provides a method of preventing, reducing or treating retinal hypoxia in a subject comprising the step of administration to an eye of the subject an effective amount of at least one of the peptides:

GlyArgArgAlaAlaProGlyArgAibGlyGly (SEQ ID NO:1)or

GlyArgArgAlaAlaProGlyArgAibGlyGly-NH_2(SEQ ID NO:2).

In a fourth aspect the invention provides a method of preventing astroglial or Mueller cell proliferative responses to an insult by administration to the eye of a subject in an effective amount of at least one of the peptides:

GlyArgArgAlaAlaProGlyArgAibGlyGly (SEQ ID NO:1)or

GlyArgArgAlaAlaProGlyArgAibGlyGly-NH_2(SEQ ID NO:2).

In another aspect, the present invention provides a method to recover or preserve the b-wave of retinal sum potentials by administration to an eye of a subject an effective amount of at least one of the peptides:

GlyArgArgAlaAlaProGlyArgAibGlyGly (SEQ ID NO:1)or

GlyArgArgAlaAlaProGlyArgAibGlyGly-NH_2(SEQ ID NO:2).

In one embodiment in each of the foregoing aspects of the invention the step of administration to the eye is by way of an intravitreal administration.

In a **further** aspect the invention provides an aqueous formulation comprising ophthalmologically or intravitreally acceptable excipients and an effective amount of at least one of the peptides:

GlyArgArgAlaAlaProGlyArgAibGlyGly (SEQ ID No:1) or

GlyArgArgAlaAlaProGlyArgAibGlyGly-NH_2 (SEQ ID NO:2).

In one embodiment, the aqueous formulation further comprises saline and,D(+)-trehalose.

In a further embodiment the aqueous formulation comprises 0.1-10mM D(+)-trehalose.

In further embodiments of the foregoing aspects of the invention the effective amount is sufficient to provide an intravitreal concentration in the range

(i)10 fM to 10 nM, or

(ii) 100 fM to 100pM.

It is to be further appreciated that the use of SEQ ID NO:1 and SEQ ID NO:2 as defined above or ophthalmic formulations as defined above can be used in for the manufacture of a medicament for preventing, reducing or treating a retinal condition as defined above in an affected eye of a subject.

In the description and claims of this specification the following acronyms, terms and phrases have the meaning provided:

"Aqueous" means consisting largely of water or dissolved in water.

"Effective amount" means an amount administered according to a dosage regimen that is effective to provide the prevention, reduction or treatment outcome.

"Insult" means an physiological event or condition that causes or brings about a hypoxic environment in, or ischemia to the eye of a subject.

"Intravitreal" means administration into the vitreous humour of a subject's eye.

"IOP" means intraocular pressure.

"Prevention" means the impeding of development of at least one symptom associated or caused by a state, disorder or disease in a subject, where the subject is not yet exhibiting any symptoms associated or caused by the state, disorder or disease.

"Reduction" means the diminishment of at least one symptom associated or caused by the state, disorder or disease being treated.

"Subject" means mammalian organisms that are capable of suffering from or are afflicted with a disease, disorder or condition associated with retinal atrophy or hypoxia. Examples of subjects include humans, dogs, cows, horses, pigs, sheep and the like.

"Treatment" means the alleviation of at least one symptom associated or caused by the state, disorder or disease being treated.

"Vitreous humour" means the jelly-like substance filling the posterior chamber of the subject eye between the lens and the retina.

In the description and claims of this specification the nucleotides and amino acids of biosequences (nucleic acids

and peptides) are identified in accordance with Tables 1 to 4 of Annex C, Appendix 2 of the PCT Administrative Instructions (as in force from January 1, 2010).

The terms "first", "second", "third", etc. used with reference to elements, features or integers of the subject matter defined in the Statement of Invention and Claims, or when used with reference to alternative embodiments of the invention are not intended to imply an order of preference.

The invention will now be described with reference to embodiments or examples and the figures of the accompanying drawings pages.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1. Shows expression of glial fibrillary protein (GFAP) as an early marker of retinal degeneration following insult (nerve fibre layer (NFL); inner nuclear layer (INL)).

Figure 2. Shows expression of glial fibrillary protein (GFAP) as an early marker of retinal degeneration following insult and administration of 100 fM of the SEQ ID NO: 2 (nerve fibre layer (NFL); inner nuclear layer (INL); outer nuclear layer (ONL)).

Figure 3. Shows expression of glial fibrillary protein (GFAP) as an early marker of retinal degeneration following insult and administration of 100 pM of the SEQ ID NO: 2 (Nerve fibre layer (NFL); inner nuclear layer (INL); outer nuclear layer (ONL)).

Figure 4. Shows cell death as detected by TUNEL following insult (arrowheads identify photoreceptors) (magnification bar is 50 µm).

Figure 5. Shows cell death as detected by TUNEL following insult and administration of SEQ ID NO:2 (arrowheads identify photoreceptors) (magnification bar is 50 µm).

Figure 6. Shows detection of the calcium binding protein parvalbumin in control retina (magnification bar is 50 µm).

Figure 7. Shows detection of the calcium binding protein parvalbumin in control retina (magnification bar is 50 µm).

Figure 8. Shows detection of the calcium binding protein parvalbumin in retina following insult by elevation of the intraocular pressure and administration of the SEQ ID NO:2 (magnification bar is 50 µm).

Figure 9. Shows detection of the calcium binding protein parvalbumin and labelling of glutamine synthetase (GS) in retina following insult by elevation of the intraocular pressure (magnification bar is 50 µm).

Figure 10. Shows labelling of glutamine synthetase (GS) in control retina (magnification bar is 50 µm).

Figure 11. Shows labelling of glutamine synthetase (GS) in retina following insult by elevation of the intraocular pressure and administration of SEQ ID NO:2 (magnification bar is 50 µm).

Figure 12. Shows double light flash ERGs taken directly after infliction of saline injection injury and at 24 hours after SEQ ID NO:2 (shown as NNZ-4945 in Figure 12) or saline intravitreal injection (after 48 hours of experimental time line lapse). Analysis paradigms comprised b-wave amplitude measurement, amplitude difference from a-wave to b-wave (PTP) and summed amplitudes.

Figure 13. Shows quantification of the astroglial (Mueller cell) response at 24 hrs after intravitreal injection of SEQ ID NO:2 (shown as NNZ-4945 in Figure 13) into the cornea that received 25µl of saline injection prior to the therapeutic intervention. Analysis consists of Glial Fibrillary Acid Protein (GFAP) density measurements within the retina.

Figure 14. Shows quantification of the astroglial (Mueller cell) response after *in vitro* hypoxia. Rats are prophylactically treated with a daily bolus of 2ng intravitreally injected SEQ ID NO:2 (shown as NNZ-4945 in Figure 14) for three subsequent days. Subsequently, retinae are extracted and subjected to 40 min of hypoxia in the presence of two concentrations of SEQ ID NO:2(shown as NNZ-4945 in Figure 14) . Analysis consists of Glial Fibrillary Acid Protein (GFAP) density measurements within the retina.

DETAILED DESCRIPTION

Tissue culture has been determined to be an ideal way to study the actions of NRPs on central nervous tissue. It allows fine control over drug concentration, time course and the analysis of the tissue on a cell-by-cell basis (Kaempf *et al* (2008); Siegel (1999)).

The specification accompanying international application no. PCT/US2008/011951 (publ. no. WO 2009/051844) discloses, as SEQ ID NO: 5, the neuroprotective activity of the amidated peptide designated SEQ ID NO: 2. These studies did not disclose the intravitreal administration of the amidated peptide.

The following experiments demonstrate that NRPs may be administered into the vitreous humour to provide a neuroprotective effect on the retina secondary to a hypoxic insult. The studies were focused on the analysis of expression of glial fibrillary protein (GFAP) as an early marker of retinal degeneration.

SEQ ID NO:2 has been shown in the studies outlined below to have high neuroprotective and astro-glial scar preventing activity in the retina. As such intravitreal administration of SEQ ID NO:2 is a therapeutic option for use in the treatment of injured hypoxic retina and other retinopathies, including high intraocular pressure associated retinopathy.

Material and methods

- *In vitro*

Retinal explants were incubated in a modified brain buffer mimicking hypoxic damage by bubbling it in 95% N_2/5% CO_2. The explants (n=4 per condition) were incubated for 40 minutes in Petri dishes individually in one of three solutions:

1. modified brain buffer alone;
2. modified brain buffer supplemented with 100 fM SEQ ID NO:2; or
3. modified brain buffer supplemented with 100 pM SEQ ID NO:2.

After 40 min the tissues were fixed by immersion in a solution with 4% paraformadehyde/0.01% glutaraldehyde for 30 minutes and processed for immunocytochemistry.

The tissue was blocked with 6% goat serum (containing 1% BSA and 0.5% Triton) and then a rabbit Glial Fibrillary Acidic Protein (GFAP) primary antibody was applied overnight. The secondary antibody was then applied.

TUNEL (Terminal deoxycleotidyl transferase dUTP Nick End Labelling, *In situ* Cell Death Detection kit, Fluorescein (ROCHE, Penzberg, Germany)) was applied to detect cell death in control and treated whole mount retinas.

- *In vivo*

To assess the *in vivo* effect of the SEQ ID NO:2, animals were anaesthetized and an ischemic insult was induced (n=5) by elevating the rat intraocular pressure (IOP) to 120mm Hg for 1.5 hours.

Subsequently, recording of retinal activity using a double light flash-induced electroretinogram (ERG) was performed to attain the base injury (reduced retinal sum potential) level. At 24 hrs after the first ERG recording. The light stimulus was calibrated with a voltage cell and elicited by twin-flashes (0.8 ms second stimulus interval) generated from a photographic flash unit (Nikon SB900 flash), via a Ganzfield sphere. Flash intensity range was from -2.9 to 2.1 log $cd.s/m^2$ and was attenuated using neutral density filters. Control waveforms were recorded by using the Scope software (AD Instruments, NZ).

The left eyes were injected with 2 pmoles of SEQ ID NO:2 (or alternatively, saline only) diluted in saline so a final vitreous chamber concentration of 50-100nM was achieved. ERG recordings were repeated after 24 hrs and subsequently the eyes were removed and processed for immunocytochemistry.

Rabbit anti-GFAP was employed to detect retinal stress and TUNEL cell detection kit was employed to detect cell death.

Results

- *In vitro*

The SEQ ID NO:2 demonstrated protective activity against cell death in this *in vitro* model of the hypoxic retina (Figures 4 and 5). Administration of the SEQ ID NO:2 reduced significantly the extent of GFAP labelling compared with the hypoxic retina (Figures 1 to 3).

There was increased expression of GFAP in the ischemic retina (Figure 1) and there was a dose-response effect of the SEQ ID NO:2, showing that 100 fM (Figure 2) was more effective at reducing the extent of GFAP expression compared with 100 pM NRP (Figure 3).

Quantification of the GFAP retinal expression after performed ischemia revealed a clearly diminished Mueller cell response in the presence of 100fM of SEQ ID NO:2 ($p<0.001$, ANOVA) when compared to saline treated control retina (see Figure 14).

- *In vivo*

In the presence of 100 fM SEQ ID NO:2 the retinal activity was not significantly different compared with a time matched ischemic control retina. However, parvalbumin labelling detecting the calcium binding protein in control retina (Figure 6 and 7) was different from the labelling in retina subjected to an elevation of the IOP (Figure 8) while restored after intravitreal administration of SEQ ID NO:2 (Figure 9).

There were no differences in glutamine synthetase (GS) labelling for all conditions (Figures 9 to 11).

Analysis of the electrical activity (sum potentials) of the retina showed that elevation of the IOP followed by 24 hours of recovery significantly reduced ($p<0.05$, 2-way ANOVA) the retinal activity (see Figure 12) which was not recovered during this time span. In contrast, SEQ ID NO:2 injection led to a significant recovery over the same time period in respect to the peak-to-peak amplitude ($p<0.001$, 2-way ANOVA) and a significant recovery of the b-wave ($p<0.05$, 2-way ANOVA).

The astroglial proliferative response of the retinal Mueller cells was significantly reduced in retinae that have been treated with a single intravitreally injected bolus of 2ng SEQ ID NO:2 that was applied 24hrs after IOP induction ($p<0.001$, ANOVA). The results are depicted in Figure 13.

Although the invention has been described with reference to embodiments or examples it should be appreciated that variations and modifications may be made to these embodiments or examples without departing from the scope of the invention.

Where known equivalents exist to specific features, such equivalents are incorporated as if specifically referred to in this specification.

REFERENCES

Kaempf *et al* (2008) *Novel organotypic culture model of adult mammalian neurosensory retina in c-culture with retinal pigment epithelium* J. of Neurosc. Methods.

Sadakata *et al* (2004) *The secretory granule-associated protein CAPS2 regulates neurotrophin release and cell survival* J. Neurosc.

Sieg and Antonic (2007) *NRPs promote adult neuronal survival/neurogenesis* In Sogos & Diana, Kerala, India.

Siegel(1999) *The golden age of retinal cell culture* Molecular Vision.

CLAIMS

1 A method of preventing, treating or reducing atrophy of a retina in a subject, comprising the step of administration to an eye of the subject an effective amount of at least one of the peptides:

GlyArgArgAlaAlaProGlyArgAibGlyGly (SEQ ID NO:1)or

GlyArgArgAlaAlaProGlyArgAibGlyGly-NH_2(SEQ ID NO:2).

2 A method as claimed in claim 1 wherein the atrophy is caused by a hypoxic or ischemic insult.

3. A method of preventing, reducing or treating intraocular pressure associated atrophy of a retina in a subject comprising the step of administration to an eye of the subject an effective amount of at least one of the peptides:

GlyArgArgAlaAlaProGlyArgAibGlyGly (SEQ ID NO:1)or

GlyArgArgAlaAlaProGlyArgAibGlyGly-NH_2 (SEQ ID NO:2).

.4 A method of preventing, reducing or treating retinal hypoxia in a subject comprising the step of administration to an eye of the subject an effective amount of at least one of the peptides:

GlyArgArgAlaAlaProGlyArgAibGlyGly (SEQ ID NO:1)or

GlyArgArgAlaAlaProGlyArgAibGlyGly-NH_2(SEQ ID NO:2).

5 A method of preventing astroglial or Mueller cell proliferative responses to an an insult by administration to the eye of an effective amount of at least one of the peptides:

GlyArgArgAlaAlaProGlyArgAibGlyGly (SEQ ID NO:1)or

GlyArgArgAlaAlaProGlyArgAibGlyGly-NH_2(SEQ ID NO:2).

6 The method of any one of claims 1 to 5 where the administration to the eye is by way of intravitreal administration.

7 An aqueous formulation comprising ophthalmologically or intravitreally acceptable excipients and an effective amount of at least one of the peptides:

GlyArgArgAlaAlaProGlyArgAibGlyGly (SEQ ID NO:1)or

GlyArgArgAlaAlaProGlyArgAibGlyGly-NH_2(SEQ ID NO:2).

8 The aqueous formulation of claim 7 wherein the formulation consists of saline comprising an effective amount of at least one of the peptides:

GlyArgArgAlaAlaProGlyArgAibGlyGly (SEQ ID NO:1)or

GlyArgArgAlaAlaProGlyArgAibGlyGly-NH_2(SEQ ID NO:2).

9) The method of any one of claims 1 to 5 wherein the effective amount is sufficient to provide an intravitreal concentration in the range 10 fM to 10 nM.

10) The method of claim 8 wherein the effective amount is sufficient to provide an intravitreal concentration in the range 100 fM to 1 nM.

11) The method of claim 9 wherein the effective amount is sufficient to provide an intravitreal concentration in the range 100 fM to 100 pM.

12) The aqueous formulation of claim 6 or claim 7 wherein the effective amount of the peptide is sufficient to provide an intravitreal concentration in the range 10 fM to 10 nM.

13) The formulation of claim 12 wherein the effective amount of the peptide is sufficient to provide an intravitreal concentration in the range 100 fM to 1 nM.

14) The formulation of claim 13 wherein the effective amount of the peptide is sufficient to provide an intravitreal concentration in the range 1 pM to 100 pM.

15) A method to recover or preserve the b-wave of retinal sum potentials by administration to an eye of a subject an effective amount of at least one of the peptides:

GlyArgArgAlaAlaProGlyArgAibGlyGly (SEQ ID NO:1)or

GlyArgArgAlaAlaProGlyArgAibGlyGly-NH_2(SEQ ID NO:2).

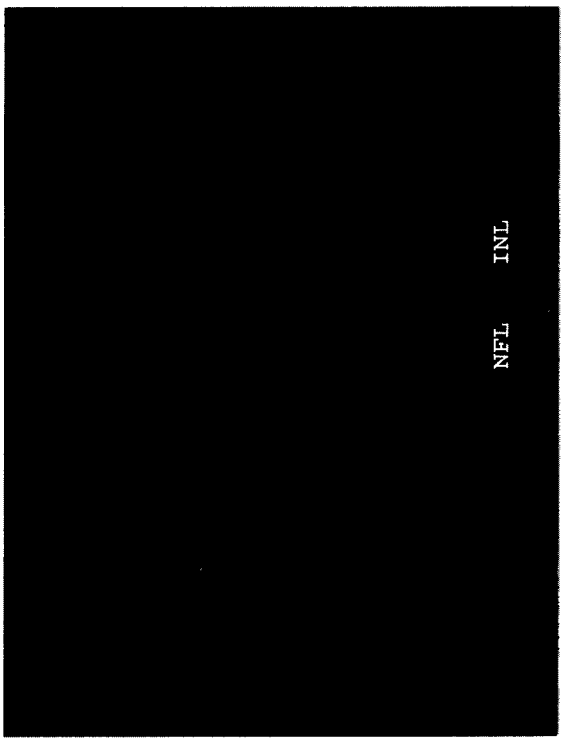


FIGURE 1

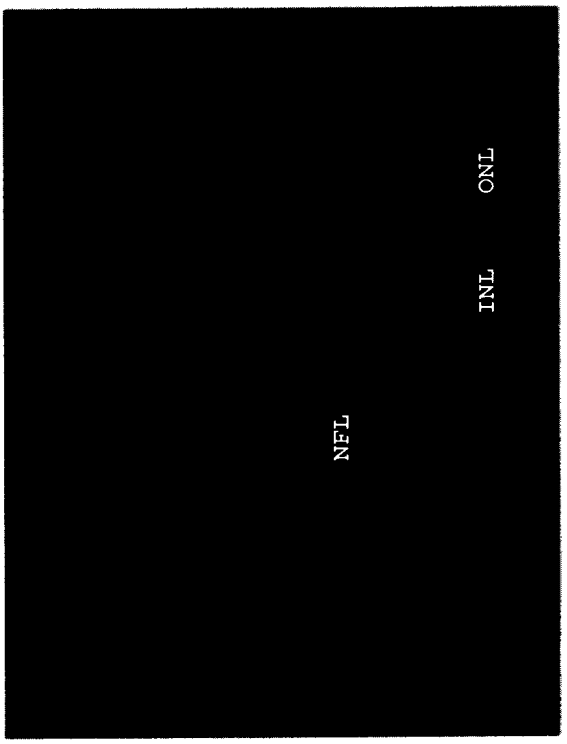


FIGURE 2

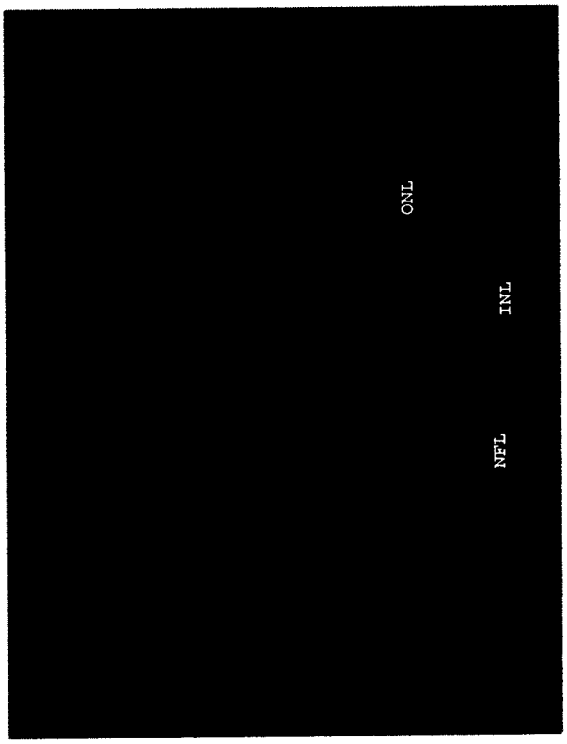


FIGURE 3

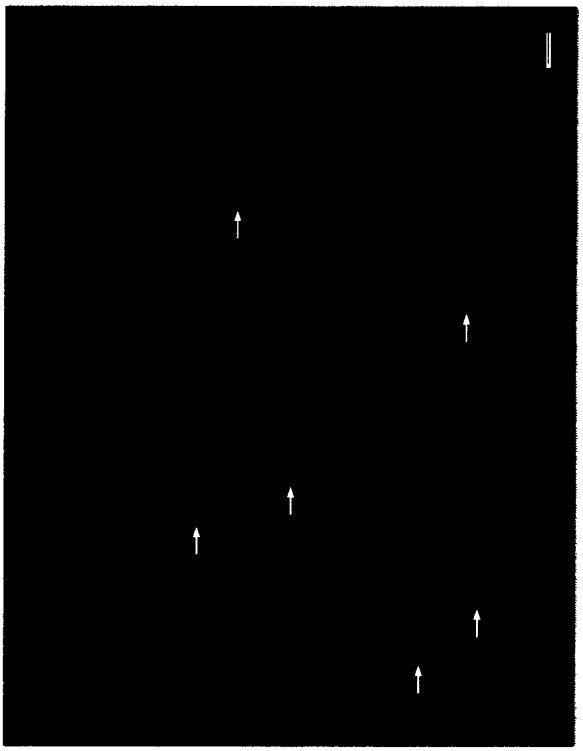


FIGURE 4

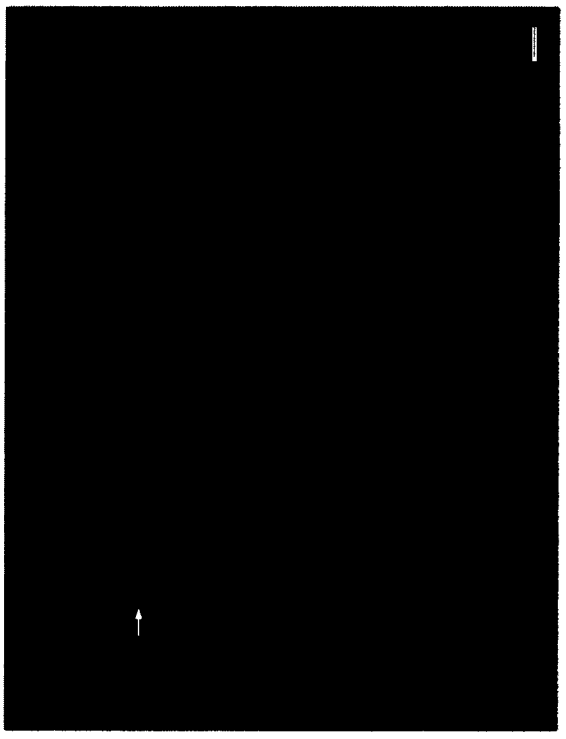


FIGURE 5

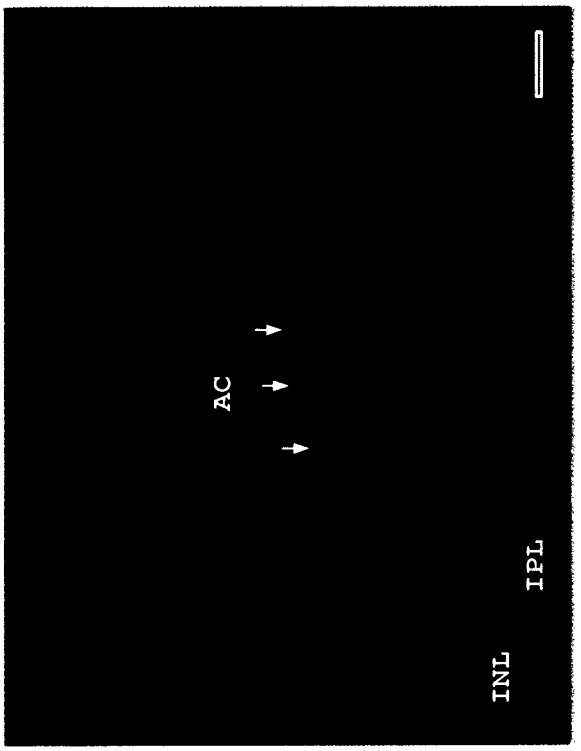


FIGURE 6

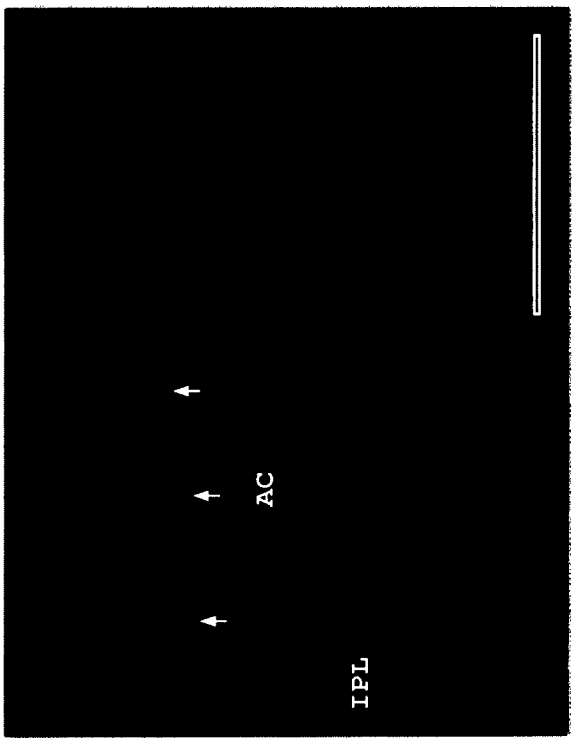


FIGURE 7

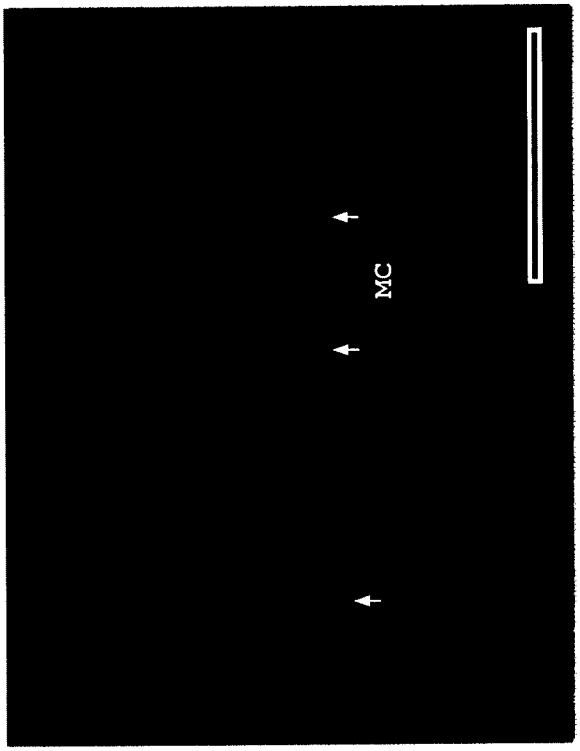


FIGURE 8

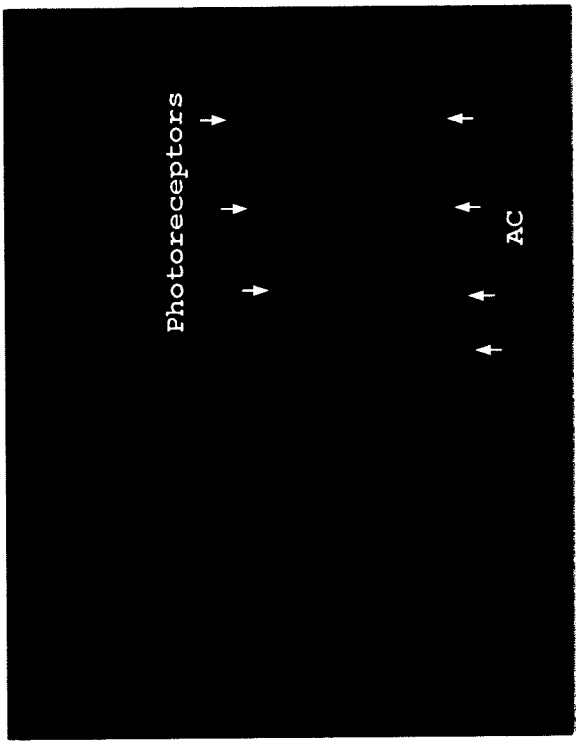


FIGURE 9

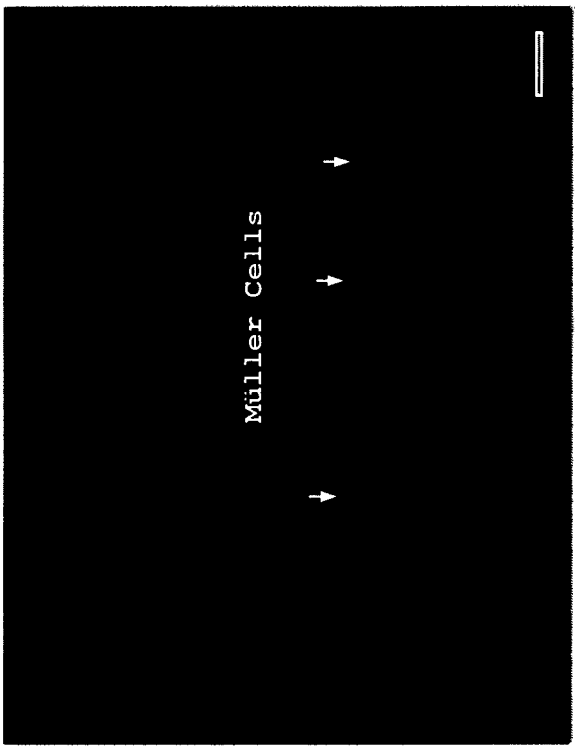


FIGURE 10

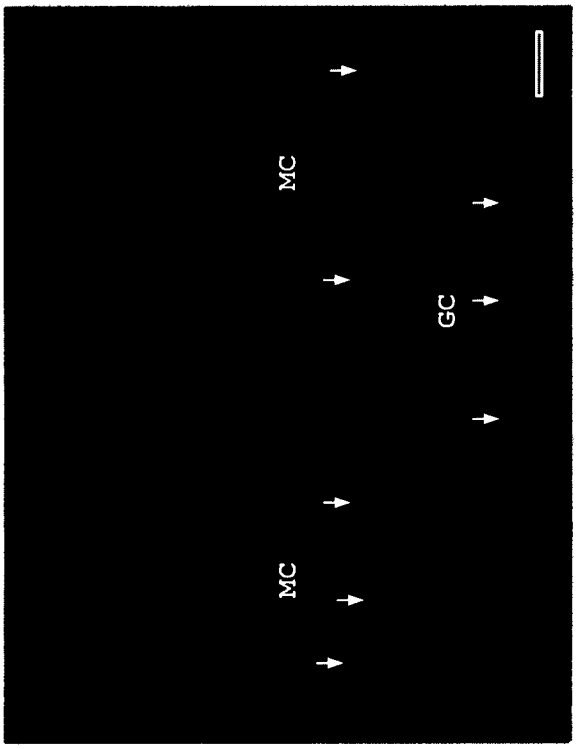


FIGURE 11

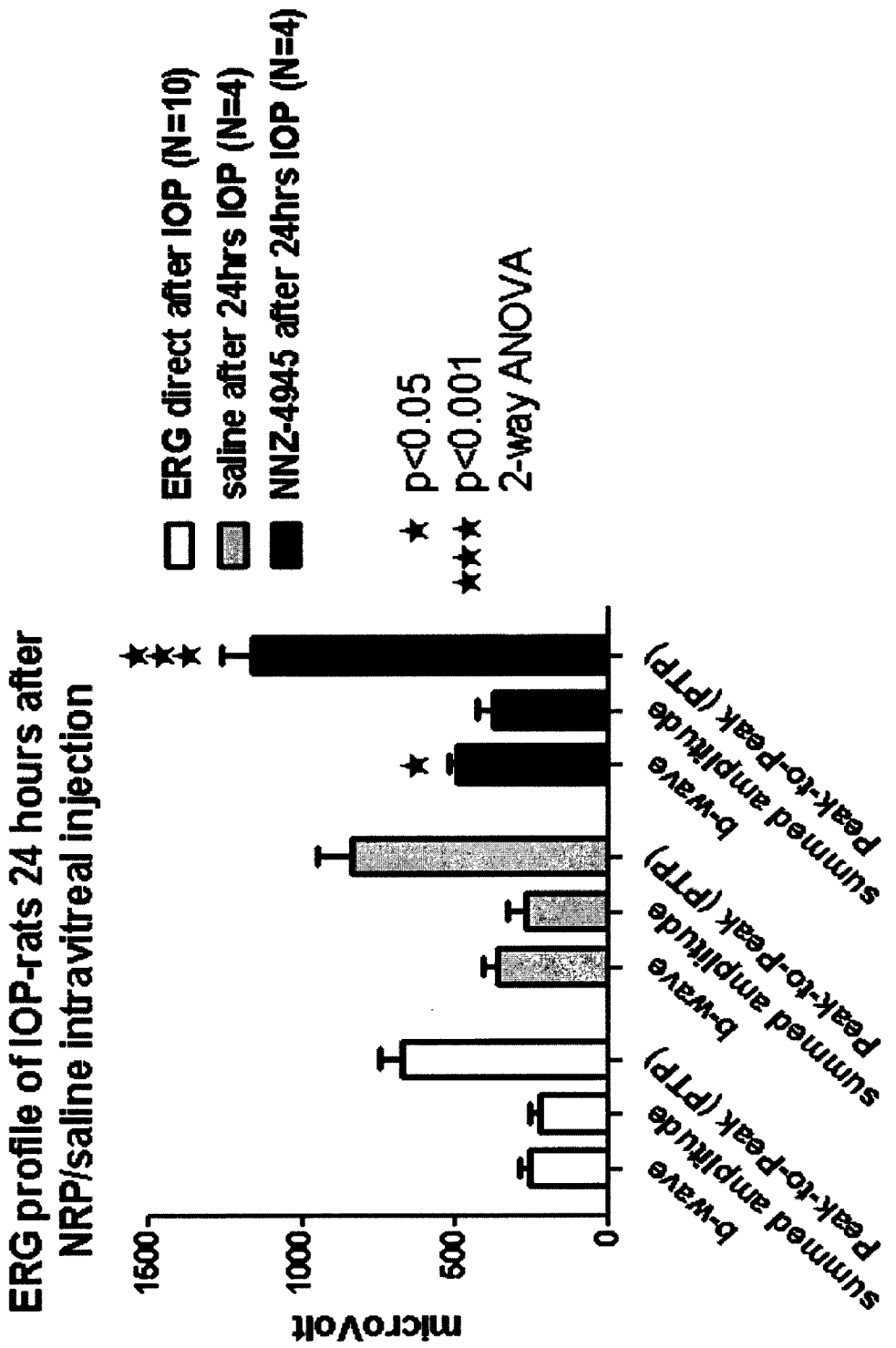
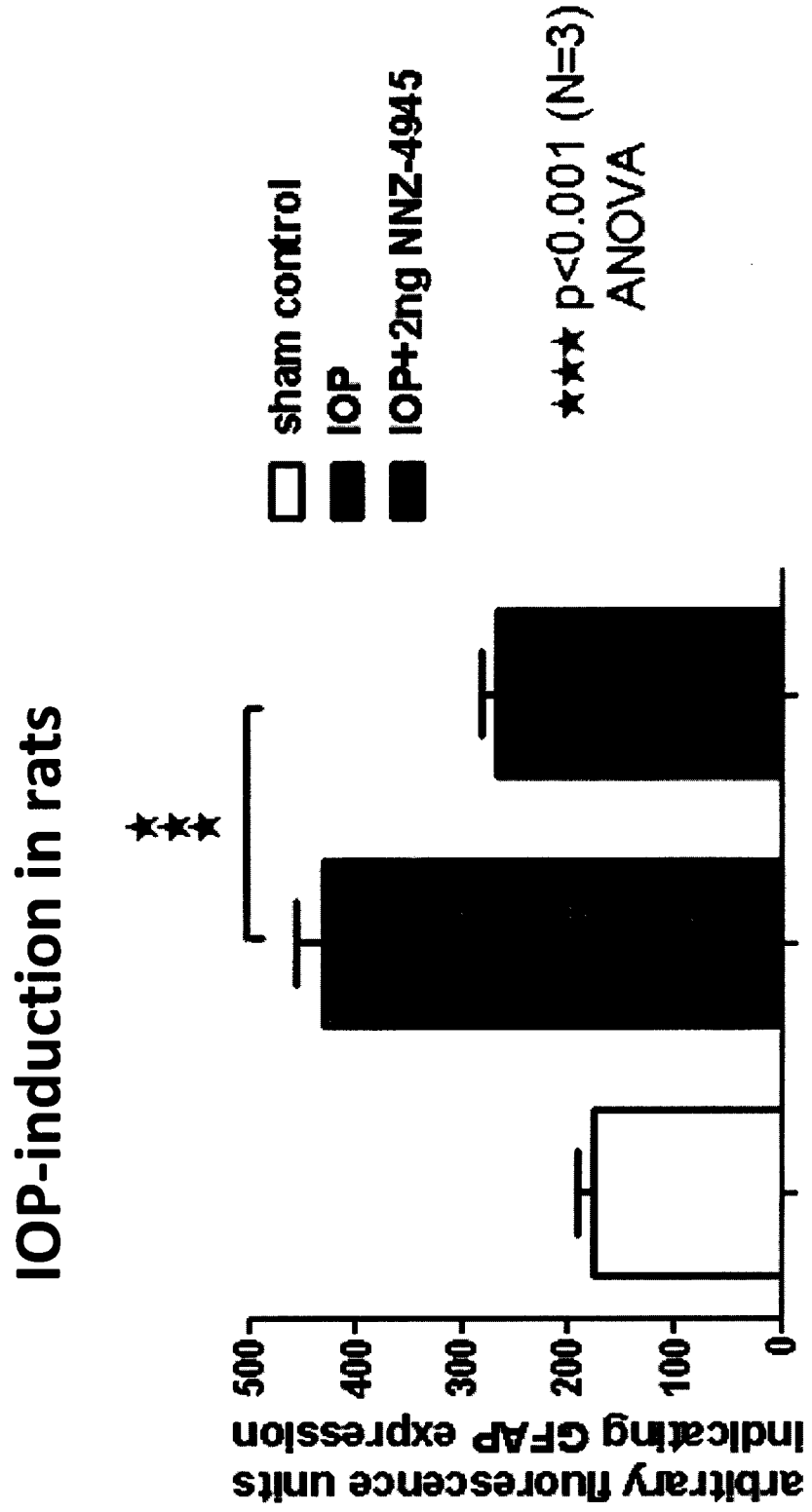

ERG profile of IOP-rats 24 hours after NRP/saline intravitreal injection
ERG direct after IOP (N=10)
saline after 24hrs IOP (N=4)
NNZ-4945 after 24hrs IOP (N=4)
★ p<0.05
★★★ p<0.001
2-way ANOVA
microVolt
1500
1000
500
0
b-wave amplitude
summed amplitude
peak-to-peak (PTP)

Figure 12

FIGURE 13


IOP-induction in rats
sham control
IOP
IOP+2ng NNZ-4945
★★★ p<0.001 (N=3)
ANOVA
★★★
arbitrary fluorescence units
indicating GFAP expression
0
100
200
300
400
500

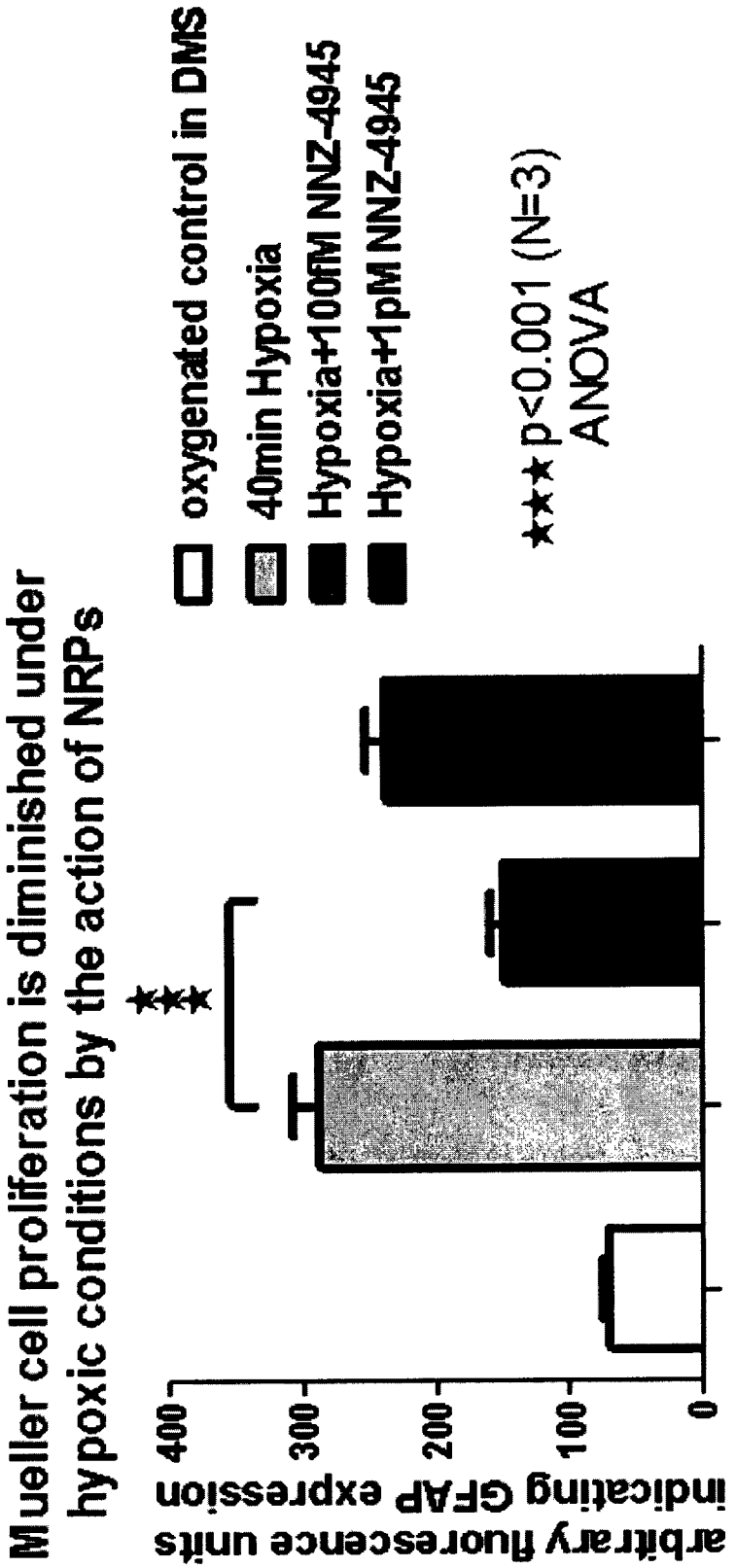

Mueller cell proliferation is diminished under hypoxic conditions by the action of NRPs
oxygenated control in DMS
40min Hypoxia
Hypoxia+100fM NNZ-4945
Hypoxia+1pM NNZ-4945
★★★ p<0.001 (N=3) ANOVA
arbitrary fluorescence units indicating GFAP expression
0
100
200
300
400
★★★

FIGURE 14